IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Christine L. Mummery	**Examiner:**	Unassigned
Serial No:	Unassigned	**Art Unit:**	Unassigned
Filed:	Herewith	**Docket:**	17360
For:	METHODS OF INDUCING DIFFERENTIATION OF STEM CELLS	**Dated:**	January 14, 2004

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CLAIM OF PRIORITY

Sir:

Applicants in the above-identified application hereby claim the right of priority in connection with Title 35 U.S.C. § 119 and in support thereof, herewith submit a certified copy of Australian Application No. PR6560, filed July 24, 2001 and Australian Application No. PS1180, filed March 18, 2002.

Respectfully submitted,

Leopold Presser
Registration No.: 19,827
Attorney for Applicant

Scully, Scott, Murphy & Presser
400 Garden City Plaza
Garden City, New York 11530
(516) 742-4343

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"Express Mail" Mailing Label Number: EV 247989835 US
Date of Deposit: January 14, 2004

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Commissioner of Patents, Alexandria, VA 22313-1450 on

Dated: January 14, 2004

Leopold Presser

Patent Office
Canberra

I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 6560 for a patent by ES CELL INTERNATIONAL PTE LTD and NETHERLANDS INSTITUUT VOOR ONTWIKKELINGSBIOLOGIE as filed on 24 July 2001.

WITNESS my hand this
Twenty-eighth day of November 2003

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **METHODS OF INDUCING DIFFERENTIATION OF STEM CELLS**

Applicants: **ES CELL INTERNATIONAL PTE LTD AND**
NETHERLANDS INSTITUUT VOOR ONTWIKKELINGSBIOLOGIE

The invention is described in the following statement:

METHODS OF INDUCING DIFFERENTIATION OF STEM CELLS

The present invention relates to methods of inducing differentiation of stem cells. In particular, the invention relates to methods of inducing differentiation of embryonic stem cells into muscle cells or vascular endothelial cells. The invention also includes cells, cell lines and culture systems used in the methods of the present invention and differentiated cells produced therefrom.

INTRODUCTION

Stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type. The process of differentiation in stem cells involves selective development of immature cells to committed and fully mature cells of various lineages. Derivatives of such lineages include, muscle, neural, skeletal, blood (hematopoietic), endothelial and epithelial cells. Differentiation of stem cells is known to be triggered by various growth factors and regulatory molecules.

During differentiation the expression of stem cell specific genes and markers are often lost and cells acquire gene expression profiles of somatic cells or their precursors. In some cases, "master" genes have been described which control differentiation versus self-renewal.

Whilst differentiation of some lineage specific stem cells can be induced with a degree of certainty, a differentiation outcome of a population of pluripotent stem cells is less predictable. Placing the cells under conditions which induce specific cell types has been one form of an attempt to regulate the differentiation outcome. These conditions include growing the cells to high or low density, changing media, introducing or removing cytokines, hormones and growth factors, creating an environment which suits differentiation toward a specific cell type, such as providing a suitable substrate.

Generally, when a stem cell culture is induced to differentiate, the differentiated population is analysed for particular cell types by expression of genes, markers or phenotypic analysis. In any case, the respective cell types may then be selectively cultured to enrich their percentage population to eventually obtain a single cell type and culture.

The induction of a specific differentiated cell type can be useful for transplantation or drug screening and drug discovery *in vitro*. Methods of inducing differention in stem cells and muscle cells produced therefrom may be used for the study of cellular and molecular biology of tissue development, for the discovery of genes, proteins, such as differentiation factors that play a role in tissue development and regeneration.

In particular, the induction of stem cells to differentiate into muscle cells (myocytes) is useful for muscle transplantation and therapeutic purposes, as well as providing potential human disease models in culture (e.g. for testing pharmaceuticals). The induction of cardiomyocyte differentiation in stem cells is especially useful in developing therapeutic methods and products for heart disease and abnormal heart conditions. However, the molecular pathways that lead to specification and terminal differentiation of specific cell types, such as myocytes, from embryonic stem cells during development are not entirely clear.

Therefore there remains a need for providing effective methods of inducing differentiation of stem cells into specific cell types, such as myocytes or endothelial cells.

SUMMARY OF THE INVENTION

In one aspect of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of an embryonic cell and/or extracellular medium of an embryonic cell, under conditions that induce differentiation of the stem cell into a specific cell type.

Preferably, the stem cell is an embryonic, human stem cell. More preferably, the stem cell is induced to differentiate into a myocyte (muscle cell), vascular endothelial cell or a haematopoietic cell. In the methods of the present invention as hereinbefore described, the embryonic cell is preferably derived from embryonic endoderm or ectoderm. More preferably, the embryonic cell is derived from extraembryonic tissue.

In the methods of the present invention the stem cell is preferably grown in the presence of an embryonic, endoderm cell and/or extracellular conditioned medium of an embryonic, endoderm cell to induce differentiation of the stem cell into a cardiomyocyte or a haematopoietic cell. More preferably, the stem cell is co-cultured in the presence of the embryonic cell. In the methods of the present invention the stem cell is preferably plated on a confluent monolayer of embryonic cells and allowed to grow in culture to induce differentiation of the stem cell.

Alternatively, the stem cell is grown in the presence of an embryonic, ectoderm cell and/or extracellular medium of an embryonic, ectoderm cell to induce differentiation of the stem cell into a skeletal muscle cell.

In yet another preferred embodiment, the stem cell is grown in the presence of an embryonic, ectoderm and/or endoderm cell, and/or extracellular medium of an embryonic, ectoderm and/or endoderm cell to induce differentiation of the stem cell into a vascular endothelial cell.

In a preferred aspect of the present invention there is provided a method of inducing muscle cell differentiation of a stem cell, the method including:

culturing a stem cell in the presence of an embryonic cell and/or extracellular medium of an embryonic cell, under conditions that induce differentiation of the stem cell into a muscle cell.

Preferably, the stem cell is grown in the presence of an embryonic, endoderm cell and/or extracellular medium of an embryonic, endoderm cell to induce

differentiation of the stem cell into a cardiomyocyte (cardiac muscle cell). More, preferably the embryonic cell is extraembryonic. The embryonic cell is preferably derived from visceral endoderm, is a cell with visceral endoderm like properties or is derived from an embryonic cell line with characteristics of visceral endoderm. More preferably, the stem cell is co-cultured in the presence of the embryonic cell.

Alternatively, the stem cell is grown in the presence of an embryonic, ectoderm cell and/or extracellular medium of an embryonic, ectoderm cell to induce differentiation of the stem cell into a skeletal muscle cell. More preferably, the embryonic cell is derived from extraembryonic tissue.

Another aspect of the present invention is an embryonic cell for use in the methods of the present invention. Preferably, the embryonic cell is derived from embryonic or extraembryonic endoderm or ectoderm. Preferably, the embryonic cell is derived from visceral endoderm or is a cell with visceral endoderm like properties. More preferably, the embryonic cell is derived from a cell line with characteristics of visceral endoderm, such as the END-2 cell line (*Mummery et al, 1985, Dev Biol. 109:402-410*).

In a further preferred aspect of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of a factor or factors derived from extracellular medium of an embryonic cell under conditions that induce differentiation of the stem cell into a specific cell type.

The present invention further provides an isolated factor or factors that is secreted from an embryonic cell according to the present invention for use in a method of inducing differentiation of a stem cell.

In yet another aspect of the invention, there is provided a differentiated cell produced according to the methods as hereinbefore described. Preferably, the differentiated cell is a cardiomyocyte, skeletal muscle cell, vascular endothelial cell or a haematopoietic cell. The present invention also provides differentiated

cells produced according to the methods of the invention that may be used for transplantation, cell therapy or gene therapy.

The present invention further provides a cell composition including a differentiated cell produced by the method of the present invention, and a carrier.

FIGURES

Figure 1A shows a phase contrast micrograph of human embryonic stem (hES) in co-culture with END-2 cells after a period of 13 days. The differentiated stem cells have mixed morphology but with a relatively high proportion of epithelial-like cells. The epithelial cells swell to fluid-filled cysts and between these cells are patches of cardiomyocytes. Cross section of the colony shown is about 2mm (40x objective). Scale bar=100µm.

Figure 1B shows a phase contrast micrograph of human embryonic stem (hES) in co-culture with END-2 cells after a period of two to three weeks. Increasing patches of beating cardiac muscle cells (cardiomyocytes; bm) are present. The beating rate observed was approximately 60 beats per minute. (20x objective). Scale bar=100µm.

Figure 2 shows cells stained positively with α-actinin, confirming that they are indeed muscle cells. Scale bar=100µm.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of an embryonic cell and/or extracellular medium of an embryonic cell, under conditions that induce differentiation of the stem cell into a specific cell type.

Stem cells usually require co-culture with a fibroblast feeder layer to maintain their undifferentiated state. Those feeder layers that do not adequately maintain this state result in stem cells loosing their undifferentiated characteristics to non-obvious phenotypes. Applicants have found that culturing stem cells with embryonic cells can provide a determining factor to the outcome of differentiated cells in culture. This control has never been seen with fibroblast cells.

The term " inducing differentiation of a stem cell" as used herein is taken to mean causing a stem cell to develop into a specific differentiated cell type as a result of a direct or intentional influence on the stem cell. Influencing factors that may induce differentiation in a stem cell can include cellular parameters such as ion influx, a pH change and/or extracellular factors, such as secreted proteins, such as but not limited to growth factors and cytokines that regulate and trigger differentiation. It may include culturing the cell to confluence and may be influenced by cell density.

In the methods of the present invention a stem cell is undifferentiated prior to culturing and is any cell capable of undergoing differentiation. The stem cell may be selected from the group including, but not limited to, embryonic stem cells, pluripotent stem cells, haematopoietic stem cells, totipotent stem cells, mesenchymal stem cells, neural stem cells, or adult stem cells.

The stem cell is preferably a human embryonic stem cell which may be derived directly from an embryo or from a culture of embryonic stem cells. For example, the stem cell may be derived from a cell culture, such as human embryonic stem cells (hES) cells (*Reubinoff et al., Nature Biotech. 16:399-404 2000)*. Whilst, the stem cell may be derived from other animals, they are most preferably human embryonic stem cells. The stem cell may be derived from an embryonic cell line or embryonic tissue. The embryonic stem cells may be cells which have been cultured and maintained in an undifferentiated state. Such cells have been described in PCT/AU99/00990, PCT/AU00/01510,

PCT/AU01/00735 and PCT/AU01/00278, the contents of which are incorporated herein by reference.

The stem cells suitable for use in the present methods may be derived from a patient's own tissue. This would enhance compatibility of differentiated tissue grafts derived from the stem cells with the patient. The stem cells may be genetically modified prior to use through introduction of genes that may control their state of differentiation prior to, during or after their exposure to the embryonic cell or extracellular medium from an embryonic cell. They may be genetically modified through introduction of vectors expressing a selectable marker under the control of a stem cell specific promoter such as Oct-4. The stem cells may be genetically modified at any stage with markers so that the markers are carried through to any stage of cultivation. The markers may be used to purify the differentiated or undifferentiated stem cell populations at any stage of cultivation.

The stem cell can be induced to differentiate into a cell type selected from the group including muscle cells, endothelial cells, such as vascular endothelial cells, epithelial cells, blood cells (haematopoietic cells) or neural cells. Preferably, the stem cell is induced to differentiate into a myocyte (muscle cell) or a vascular endothelial cell. More preferably, the stem cell is induced to differentiate into a cardiomyocyte or a skeletal muscle cell.

In a preferred embodiment of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of an embryonic cell under conditions that induce differentiation of the stem cell into a specific cell type.

The embryonic cell used in the present method includes an embryonic cell derived from an embryo or a cell derived from extraembryonic tissue. The term "embryo" is defined as any stage after fertilisation up to 2 weeks post conception in mammals. It develops from repeated division of cells and includes the stages of a blastocyst stage which comprises an outer trophectoderm and an inner cell mass (ICM). The embryo may be an *in vitro*

fertilised embryo or it may be an embryo derived by transfer of a somatic cell or cell nucleus into an enucleated oocyte preferably of human or non-human origin. Extraembryonic tissue includes cells produced by the embryo that make up the placenta.

In a preferred embodiment of the invention, the embryonic cell is derived from embryonic, preferably extraembryonic, endoderm or ectoderm. More preferably, the embryonic cell is derived from visceral endoderm tissue or visceral endoderm like tissue isolated from an embryo. Preferably visceral endoderm may be isolated from early postgratulation embryos, such as mouse embryo (E7.5). Visceral endoderm or visceral endoderm like tissue can be isolated as described in *Roelen et al, 1994 Dev. Biol. 166:716-728.* Characteristically the visceral endoderm may be identified by expression of alphafetoprotein and cytokeratin ENDO-A). The embryonic cell may be an embryonic carcinoma cell, preferably one that has visceral endoderm properties.

The embryonic cell may be derived from a cell line or cells in culture. The embryonic cell may be derived from an embryonic cell line, preferably a cell line with characteristics of visceral endoderm, such as the END-2 cell line (*Mummery et al, 1985, Dev Biol. 109:402-410*). The END-2 cell line was established by cloning from a culture of P19 EC cells treated as aggregates in suspension (embryoid bodies) with retinoic acid then replated (*Mummery et al, 1985, Dev Biol. 109:402-410).* The END-2 cell line has characteristics of visceral endoderm (VE), expressing alpha-fetoprotein (AFP) and the cytoskeletal protein ENDO-A. Accordingly it is most preferred that the embryonic cell is derived from the END-2 cell line. These cells have been found to be particularly useful for inducing differentiation of a human stem cell to a cardiomyocyte (cardiac muscle cell).

The embryonic cell may be an ectoderm cell, which can be isolated according to methods described in *Roelen et al 1994, Dev. Biol. 166:716-728.* Ectoderm cells are known to express oct-4 and have alkaline phosphatase activity and they also have SSEA-1 on their cell surface. Therefore, ectoderm cells can be identified and isolated based on the above characteristics. Ectoderm cells may

secrete (growth) factors that induce differentiation to skeletal muscle or vascular endothelial cells. It is preferred that the ectoderm cells are derived from E7.5, embryonic mouse tissue.

Accordingly, in another aspect of the present invention there is provided an embryonic cell for use in the methods of the present invention. Preferably, the embryonic cell is derived from embryonic endoderm or ectoderm as discussed above.

In the present invention and methods as hereinbefore described, the stem cell and embryonic cell are cultured to induce differentiation into a specific cell type. Preferably, the stem cell and embryonic cell are co-cultured *in vitro*. This typically involves introducing the stem cell to an embryonic cell monolayer produced by proliferation of the embryonic cell in culture. Preferably, the embryonic cell monolayer is grown to confluence and the stem cell is allowed to grow in the presence of extracellular medium of the embryonic cells for a period of time sufficient to induce differentiation of the stem cell to a specific cell type. Alternatively, the stem cell may be allowed to grow in culture containing the extracellular medium of the embryonic cell(s), but not in the presence of the embryonic cell(s). The embryonic cells and stem cells could be separated from each other by a filter or an acellular matrix such as agar.

In the methods of the present invention the stem cell is preferably plated on a monolayer of embryonic cells and allowed to grow in culture to induce differentiation of the stem cell. More preferably, the monolayer is confluent and is mitogenically inactive

Conditions for obtaining differentiated embryonic stem cells are those which are non-permissive for stem cell renewal, but do not kill stem cells or drive them to differentiate exclusively into extraembryonic lineages. A gradual withdrawal from optimal conditions for stem cell growth favours differentiation of the stem cell to specific cell types. Suitable culture conditions may include the addition of DMS, retinoic acid or BMPs in co-culture which could increase differentiation rate and/or efficiency.

The cell density of the embryonic cell layer affects its stability and performance. The embryonic cells should preferably be confluent. More preferably, the embryonic cells are grown to confluence and are then exposed to an agent which prevents further division of the cells, such as mitomycin C. The embryonic monolayer layer is preferably established 2 days prior to addition of the stem cell(s). The stem cells are preferably dispersed and then introduced to a monolayer of embryonic cells. More preferably, the stem cells and embryonic cells are co-cultured for a period of two to three weeks until a substantial portion of the stem cells have differentiated. Preferably, the stem cell is induced to differentiate into a myocyte (muscle cell) including cardiomyocytes and skeletal muscle cells, a vascular endothelial cell or a haematopoietic cell. It is preferred that the embryonic cell is derived from extraembryonic tissue and more preferably from embryonic endoderm or ectoderm.

In another preferred embodiment of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of extracellular medium of an embryonic cell under conditions that induce differentiation of the stem cell into a specific cell type.

The term "extracellular medium of an embryonic cell" as used herein is taken to mean conditioned medium produced from growing an embryonic cell as herein described in a medium for a period of time so that extracellular factors, such as secreted proteins, produced by the embryonic cell are present in the conditioned medium. The medium can include components that encourage the growth of the cells, for example basal medium such as Dulbecco's minimum essential medium, Ham's F12, or foetal calf serum.

In an even further preferred aspect of the present invention there is provided a method of inducing differentiation of a stem cell, the method including:

culturing a stem cell in the presence of a factor or factors derived from extracellular medium of an embryonic cell under conditions that induce differentiation of the stem cell into a specific cell type.

The extracellular medium preferably includes cellular factors, such as secreted proteins, that are capable of inducing differentiation of a stem cell. Such secreted proteins will typically bind receptors on a cell surface to trigger intracellular pathways which can initiate differentiation of the cell. Examples of suitable extracellular factors include Ihh and BMP2 as described in *Dyer et al 2001, Dev. 128: 1717-1730.*

In another aspect of the present there is provided an isolated factor or factors that is/are secreted from an embryonic cell according to the present invention for use in a method of inducing differentiation of a stem cell. Suitable isolated factors may be selected by their ability to induce differentiation of a stem cell. For example, culture cell assay systems can be used to identify protein fractions and specific factors that are capable of inducing differentiation of a stem cell. The factors may include secreted proteins that are present in the extracellular medium of an embryonic cell. Suitable proteins may be extracted and purified by conventional methods known to those skilled in the field.

In another preferred aspect of the present invention there is provided a method of inducing muscle cell differentiation of a stem cell, the method including:

culturing a stem cell in the presence of an embryonic cell and/or extracellular medium of an embryonic cell, under conditions that induce differentiation of the stem cell into a muscle cell.

In a preferred embodiment the stem cell is induced to differentiate in to a cardiomyocyte cell. The applicants have found that culturing the stem cell with embryonic, endoderm cells causes a preferential induction of differentiation toward specific cell types, in particular toward muscle cells. It is most preferred that this combination of stem cell and embryonic endoderm cells induces differentiation toward cardiomyocytes. It is preferred that the stem cell is human, preferably a human embryonic stem cell (hES). More preferably, the stem cell is co-cultured with the embryonic cell. This is typically achieved by introducing dispersed stem cells to a culture medium with a monolayer of

suitable embryonic cells. More preferably, the monolayer is of confluent embryonic cells.

In an even further preferred embodiment the embryonic cell is an endoderm cell derived from visceral endoderm or is an embryonic cell with visceral endoderm properties. More preferably, the visceral endoderm cells are derived from E7.5 mouse embryo. The embryonic cell may be an embryonic carcinoma cell, preferably one that has visceral endoderm properties. More preferably, the embryonic cell is derived from a cell line or cells in culture. The embryonic cell may be derived from an embryonic cell line, preferably a cell line with characteristics of visceral endoderm, such as the END-2 cell line (*Mummery et al, 1985, Dev Biol. 109:402-410*). More, preferably the embryonic cell is derived from extraembryonic tissue and more preferably is derived from visceral endoderm. Endoderm cells are typically adjacent to sites of heart formation in vertebrates. In individuals where endoderm differentiation is defective or absent, the heart develops abnormally.

In order to induce differentiation of the stem cell to a cardiomyocyte it is preferable to introduce the stem cell to an extraembryonic, endoderm cell monolayer in culture. The monolayer is produced by proliferation of the embryonic cell derived from embryonic endoderm, more preferably extraembryonic endoderm. The embryonic endoderm is preferably extraembryonic, visceral endoderm. More preferably, the cell monolayer is produced by END-2 cells. It is preferred that the embryonic cells are cultured and passaged before allowing them to grow to form a monolayer. The monolayer is preferably grown to confluence in a suitable medium, such as DMEM or M16 medium. The monolayer may then be treated with certain agents to prevent further division of the cells. For instance, the monolayer can be treated with mitomycin and then the stem cell can be plated on the mitogenically inactive monolayers.

The stem cells are allowed to grow in the presence of extracellular medium of the endoderm cells for a period of time sufficient to induce differentiation of the stem cell to a cardiomyocyte, that is most preferably beating. Most preferably,

the co-culturing is carried out for about two to three weeks and the medium is preferably replaced regularly such as every 5 to 6 days with fresh medium.

Alternatively, the stem cell may be allowed to grow in culture containing the extracellular medium of the endoderm cells, but not in the presence of the endoderm cells. Therefore, the stem cell may be grown in the presence of extracellular medium of an embryonic, endoderm cell to induce differentiation of the stem cell into a muscle cell such as a cardiomyocyte (cardiac muscle cell).

In the methods of the present invention the cardiomyocyte cells produced are preferably beating. Cardiomyocytes are a differentiated cell type derived from stem cells. Muscle cells, including cardiomyocytes, can be fixed and stained with α-actinin antibodies to confirm muscle phenotype. α-troponin, α-tropomysin and α-MHC antibodies also give characteristic muscle staining. Ion channel characteristics and action potentials of muscle cells can be determined by patch clamp, electrophysiology and RT-PCR.

Stem cells from which cardiomyocytes are to be derived can be genetically modified to bear mutations in, for example, ion channels (this causes sudden death in humans). Cardiomyocytes derived from these modified stem cells will thus be abnormal and yield a culture model for cardiac ailments associated with defective ion channels. This would be useful for basic research and for testing pharmaceuticals. Likewise, models in culture for other genetically based cardiac diseases could be created. Cardiomyocytes produced in the present methods can also be used for transplantation and restoration of heart function.

In yet another preferred aspect there is provided a method of inducing differentiation of a stem cell to a skeletal muscle cell, the method including:

culturing a stem cell in the presence of an embryonic ectoderm cell and/or extracellular medium of an embryonic ectoderm cell, under conditions that induce differentiation of the stem cell into a skeletal muscle cell.

Ectoderm cells can be isolated according to methods described in *Roelen et al 1994, Dev. Biol. 166:716-728.* Ectoderm cells are known to express oct-4 and

have alkaline phosphatase activity and they also have SSEA-1 on their cell surface. Therefore, ectoderm cells can be identified and isolated based on the above characteristics. Ectoderm cells may secrete (growth) factors that induce differentiation to skeletal muscle. It is preferred that the ectoderm cells are derived from E7.5, embryonic mouse tissue. More preferably, the ectoderm cells are co-cultured with the stem cells using similar methods as discussed earlier. An ectoderm monolayer is preferably established in culture and preferably dispersed stem cells are introduced to the culture for a period of time sufficient to induce differentiation of the stem cells to skeletal muscle cells.

Skeletal muscle cells are typically elongated, multinucleate cells that "twitch" and express MF20. When skeletal muscle cells are stained with α-actinin they normally produce a striped staining pattern. Skeletal muscle cells produced by the methods of the present invention can be used for transplantation in the treatment of muscle disease.

In yet another preferred aspect there is provided a method of inducing differentiation of a stem cell to a vascular endothelia cell, the method including:

culturing a stem cell in the presence of an embryonic ectoderm and/or endoderm cell, and/or extracellular medium of an embryonic, ectoderm and/or endoderm cell to induce differentiation of the stem cell into a vascular endothelial cell.

More, preferably the embryonic cell is derived from extraembryonic ectoderm and/or endoderm tissue. Preferably, embryonic tissue is derived form embryonic E7.5 mouse. The embryonic ectoderm or endoderm cell may be obtained as previously discussed. The first vascular network in the embryo forms adjacent to visceral extraembryonic endoderm in the yolk sac, which produce factors affecting endothelial cells like TGFβ and VEGF. Therefore, more preferably the embryonic cell used in the above method is derived from visceral extraembryonic endoderm or is a cell with visceral cell like properties. The embryonic endoderm and/or ectoderm cell is preferably co-cultured with the stem cell using methods previously discussed. However, VEGF may be added to the culture medium to promote vascular endothelial cell growth in culture.

Vascular endothelial cells produced by the method of the invention can be identified by being capable of forming vascular networks sometimes containing blood. The vascular endothelial cells typically express receptors for VEGF, stain for PE-CAM, VE-CAM and von Willebrand factor. The vascular endothelial cells produced by the methods of the present invention would be useful as models for genetically based vascular disease. An example could be human hereditary telangiectasia, where patients have mutations in TGF-β receptors and a chronic bleeding syndrome. It is difficult to isolate and maintain long-term cells from patients with this disease to understand the pathology. Therefore, genetically modified stem cells induced to differentiate to vascular endothelial cells can provide a useful culture model. In addition, the vascular endothelial cells produced by the present methods can be used for transplantation and/ or a route for delivery of gene therapy.

In yet another aspect of the invention, there is provided a differentiated cell produced according to the methods as hereinbefore described. Preferably, the differentiated cell is a cardiomyocyte, skeletal muscle cell, vascular endothelial cell or a haematopoietic cell. The present invention also provides differentiated cells produced according to the methods of the invention that may be used for transplantation, cell therapy or gene therapy. The differentiated cells may be used as a source for isolation or identification of novel gene products including but not limited to growth factors, differentiation factors or factors controlling tissue regeneration, or they may be used for the generation of antibodies against novel epitopes.

The differentiated cells produced according to the methods of the present invention may be clonally expanded. A specific differentiated cell type can be selectively cultivated from a mixture of other cell types and subsequently propagated. Specific differentiated cell types that are clonally expanded can be useful for various applications such as the production of sufficient cells for transplantation therapy, for the production of sufficient RNA for gene discovery studies etc. The differentiated cells may be used to establish cell lines according to conventional methods.

The differentiated cells produced according to the methods of the present invention may be genetically modified. For instance, a genetic construct may be inserted to a differentiated cell at any stage of cultivation. The genetically modified cell may be used after transplantation to carry and express genes in target organs in the course of gene therapy.

The differentiated cells produced according to the methods of the present invention may be preserved or maintained by any methods suitable for storage of biological material. Effective preservation of differentiated cells is highly important as it allows for continued storage of the cells for multiple future usage. Traditional slow freezing methods, commonly utilised for the cryo-preservation of cell lines, may be used to cryo- preserve differentiated cells.

The present invention further provides a cell composition including a differentiated cell produced by the method of the present invention, and a carrier. The carrier may be any physiologically acceptable carrier that maintains the cells. It may be PBS or other minimum essential medium known to those skilled in the field. The cell composition of the present invention can be used for biological analysis or medical purposes, such as transplantation. In addition, the cell composition of the present invention can be used in methods of treating diseases or conditions, such as cardiac disease.

The present invention will now be more fully described with reference to the accompanying examples and drawings. It should be understood, however that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1: Differentiation of human embryonic stem (hES) cells into cardiomyocytes:

(a) Co-culturing of hES cells with END-2 cells

Human embryonic stem cells (hES cells) *Reubinoff et al, Nature Biotech. 16:399-404)* were co-cultured with END-2 cells ((*Mummery et al, 1985, Dev Biol. 109:402-410*). The END-2 cells are grown routinely in a 1:1 ratio of Dulbecco's minimum essential medium (DMEM) and Ham's F12 medium (DF) with 7.5% FCS. Cells were then passaged twice weekly, 1:5 using trypsin/EDTA (0.125%w/v; 50mM resp). The hES cells were cultured in DMEM with 20% FCS, 0.1mM β-mrcaptoethanol, 1% non-essential amino acids, 2mM glutamine plus antibiotics (pen/strep) on mitomycin (10μg/ml) treated embryonic feeder cells. HES were subcultured by treating with dispase and mechanical slicing of individual colonies into 6-10 pieces followed by transfer of the pieces to new feeder cells.

To initiate co-cultures, confluent cultures of END-2 cells were first passaged 1:10 on to gelatin-coated glass coverslips or tissue culture plastic wells in DMEM with 7.5% FCS and grown for 3 days to confluency. Monolayers were then treated with 10μg/ml mitomycin C for 3 hours, washed 3 times in phosphate-buffered saline (PBS) without Ca and Mg, and hES medium added. The hES cells were then dispersed using 10mg/ml dispase for 3-5 minutes, followed by gentle agitation in a pipette to yield a suspension containing small cell clumps of approximately 10-50 cells. These suspensions were then plated on the mitogenically inactive END-2 monolayers, for 2-3 weeks, in hES complete medium. The medium was replaced every 5-6 days with fresh hES medium .

(b) Co-culturing of hES c lls with visceral endoderm c lls

Visceral endoderm cells were isolated from the three germ layers of gastrulating mouse embryos at E7.5 (where E0.5 is noon on the day of the vaginal plug, as described previously using dispase (*Roelen et al, 1994, Dev. Biol. 166:716-728*). The separated germ layers were plated on to poly-L-lysine coated culture dishes in M16 medium and allowed to attach overnight. The next day, M16 was replaced by hES complete medium, and on day 3 after germ cell isolation, pieces of undifferentiated cell "transfers" were plated on to the attached endodermal and ectodermal cell from the mouse embryo. Cultures were then grown for 2 to 3 weeks and medium refreshed every 5-6 days.

(c) Analysis of co-culture experiments (a) and (b)

The cultures described in (a) and (b) above were scored for the presence of areas of beating muscle from 10 days onwards.

Immunofluorescence.

The cultures were then fixed after areas of beating muscle became evident in 2% paraformaldehyde for 30 min, washed 3 times and stored in PBS until use. The cultures were then stained to verify the muscle phenotype using α-actinin antibodies (monoclonal anti-α-actinin (sarcomeric) clone EA-53, dilution 1:1000, Sigma; secondary antibody: goat antimouse-IgG-Cy3).

(d) Results of co-culture experiments (a) and (b)

(i) hES – END-2 co-culture.

During the first week of co-culture, the clumps of cells gradually spread and differentiate to cells with mixed morphology but with a relatively high proportion of epithelial-like cells. By the second week, these swell to fluid-filled cysts (see Figure 1). Between these, distinct patches of cells become evident which begin to beat a few days later. Between 12 and 21 days, increasingly more of these beating patches appear. There is no apparent difference between glass and tissue culture substrates, both yielding beating muscle as demonstrated in 3 independent experiments that indicated 15-20% of the wells containing one or

more areas of beating muscle. Beating rate is approximately 60 beats per minute and highly temperature sensitive. These cells stain positively with α-actinin, confirming that they are indeed muscle cells (Figure 2).

(ii) hES – E7.5 endoderm co-culture.

During the first week of culture, the hES pieces placed on top of the endoderm gradually begin to spread and flatten and on day 12, the first areas of beating muscle cells become evident. This is not accompanied by the extensive cyst formation observed in the END-2 co-cultures but areas resembling vascular endothelial cell networks do appear at the edges of the culture.

Example 2: Differentiation of human embryonic stem (hES) cells into skeletal muscle cells:

Human stem cells (hES) as used in Example 1 were placed on ectoderm isolated from E7.5 day embryos (E0.5 is day of plug). With sharpened tweezers and tungsten needles, the embryos were prepared out of the decidua and kept on ice in HEPES- buffered DMEM containing 10% FCS. After removing Reichert's membrane, the embryonic and extraembryonic parts of the conceptus were separated with tungsten needles. The node and primitive streak were removed and the embryonic part incubated in 2.5% pancreatin and 0.5% trypsin in PBS on ice for 8 min. After incubation, the embryos were transferred to HEPES-buffered DMEM containing 10% FCS on ice. The ectoderm, endoderm and mesoderm could then be cleanly isolated using tungsten needles.

The hES cells initially resemble those on endoderm but by day 18 there are areas of highly elongated, twitching cells that resemble skeletal muscle. There are no areas reminiscent of beating cardiac muscle although vascular networks (Vascular endothelial cells) are evident.

Example 3: Differentiation of human embryonic stem (hES) cells into vascular endothelial cells:

Human stem cells (hES) as used in Example 1 were placed on ectoderm and/or endoderm cells. Co-culture conditions of hES with ectoderm or endoderm were as above. Vascular endothelial cells in networks accompanied differentiation to other somatic cell types.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The discussion of prior art documents, acts, devices and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the filing date of this application.

Finally, the invention as hereinbefore described is susceptible to variations, modifications and/or additions other than those specifically described and it is understood that the invention includes all such variations, modifications and/or additions which may be made it is to be understood that various other modifications and/or additions which fall within the scope of the description as hereinbefore described.

DATED: 24 July, 2001

PHILLIPS ORMONDE & FITZPATRICK

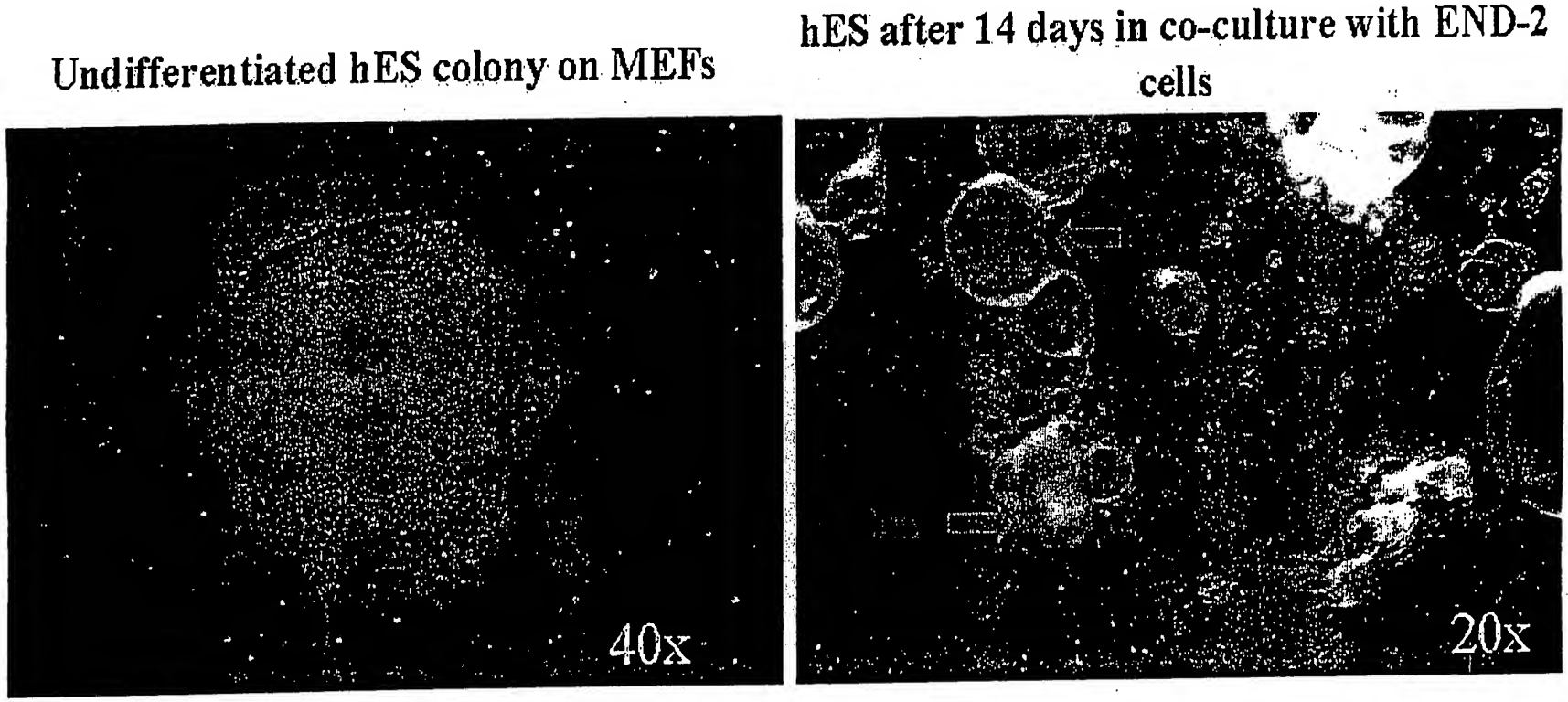
Attorneys for:

ES CELL INTERNATIONAL PTE LTD and
NETHERLANDS INSTITUUT VOOR ONTWIKKELINGSBIOLOGIE

David B Fitzpatrick

FIGURE 1A

FIGURE 1B

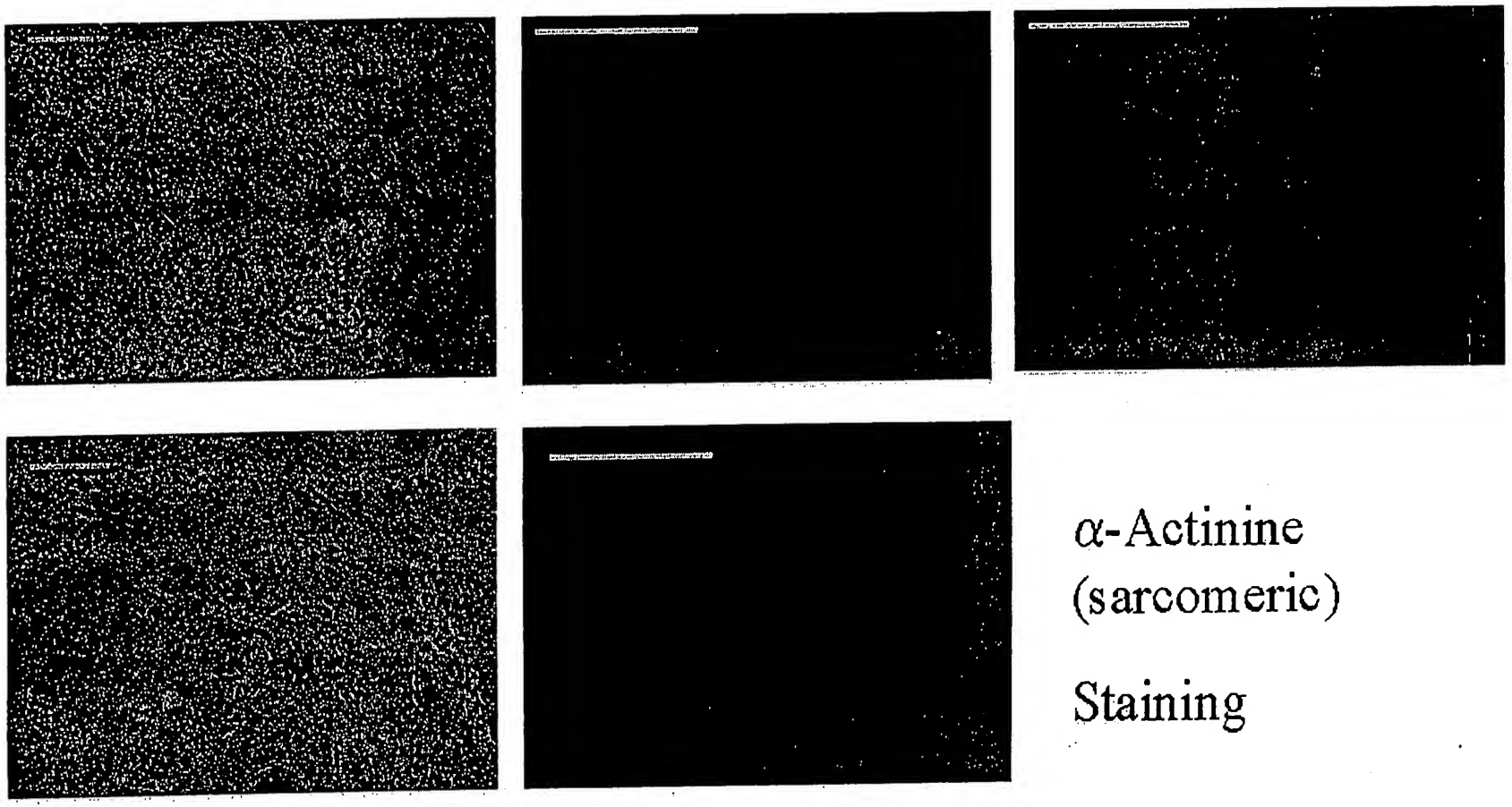


hES cells differentiate in co-culture with END-2 cells

Bm shows an area of beating muscle; cy shows one on many cystic embryonic endoderm structures

FIGURE 2

hES cells



Phase contrast: area of beating muscle

Fluorescent image of same area

α-Actinine (sarcomeric) Staining